

Evaluation of Different Cytomegalovirus (CMV) DNA PCR Protocols for Analysis of Dried Blood Spots from Consecutive Cases of Neonates with Congenital CMV Infections[▽]

Oriane Soetens,¹ Christelle Vauloup-Fellous,² Ina Foulon,³ Pascal Dubreuil,² Ben De Saeger,¹ Liliane Grangeot-Keros,² and Anne Naessens^{1*}

Department of Microbiology, UZ Brussel, Laarbeeklaan 101, Brussels 1090, Belgium¹; Department of Microbiology and Immunology, Assistance Publique-Hôpitaux de Paris, Antoine Bécère Hospital, 157 av. de la porte de Trivaux 92141 Clamart, Faculty of Medicine Paris-Sud, France²; and Department of ENT, UZ Brussel, Laarbeeklaan 101, Brussels 1090, Belgium³

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Two protocols for the extraction of cytomegalovirus (CMV) DNA and two methods for the amplification of CMV DNA in dried blood spots were evaluated for the retrospective diagnosis of congenital CMV infection. During the period from 1996 to 2006, a urine screening program detected 76 congenitally infected neonates. Stored Guthrie cards with blood from 55 cases and 12 controls were tested. Two spots of dried blood were cut from each card and evaluated in two centers. CMV DNA was extracted from a whole single spot. Center 1 used phenol-chloroform extraction and ethanol precipitation followed by a conventional PCR. Center 2 used the NucliSens easyMAG automated DNA/RNA extraction platform (bioMérieux) followed by a real-time PCR. For evaluation of the extraction method, DNA extracted from each blood spot was evaluated by the amplification method used by the collaborating center. The sensitivities were 66% for center 1 and 73% for center 2. None of the controls were positive. A sensitivity as high as 82% could be obtained by combining the most sensitive extraction method (the phenol-chloroform procedure) with the most sensitive PCR method (real-time PCR). The detection rate was not influenced by the duration of storage of the spots. The sensitivity was higher with blood from congenitally infected cases due to a primary maternal CMV infection, regardless of the protocol used. However, the difference reached significance only for the least-sensitive protocol ($P = 0.036$).

Cytomegalovirus (CMV) infection during pregnancy can be transmitted to the fetus, resulting in a congenital infection. Congenital CMV infection (cCMV) occurs in 0.2 to 2.2% of live-born infants. An incidence of 0.62% is found in the Brussels, Belgium, region (11).

The standard diagnosis of cCMV relies on the isolation of the virus from urine or saliva collected in the first 2 weeks of life. The diagnosis of cCMV infection in children older than 2 weeks cannot be made or excluded on the basis of viral isolation. Blood is routinely collected from neonates in the first 2 weeks of life and is stored as dried blood spots (DBSs) on Guthrie cards. The detection of CMV DNA on these stored DBSs could be an opportunity to diagnose cCMV during later life, when symptoms suggestive of cCMV develop (3).

The aim of this study was to test the clinical sensitivity of CMV DNA PCR with DBSs from consecutive cases of neonates with cCMV infections. We evaluated two methods for the extraction of CMV DNA and two methods for the amplification of CMV DNA from DBSs on Guthrie cards for the retrospective diagnosis of cCMV.

MATERIALS AND METHODS

The study protocol was approved by the Committee of Medical Ethics of the UZ Brussel.

cCMV infections. From 1996 to 2006 a neonatal screening program (11) detected cCMV in 76 newborns at the Universitair Ziekenhuis Brussels. The diagnosis of cCMV infection was made by isolation of the virus from urine within 7 days of birth. Blood from the first consecutive 57 cases and from the last 4 cases was retrieved and placed on Guthrie cards (no. 903 blood collection cards; Schleicher & Schuell), which were stored at room temperature. Sufficient dried blood was present on 55 of the cards, enabling testing at two centers.

The mean duration of storage of the cards was 72.9 ± 31 months (range, 0.5 to 130 months).

Two infants were symptomatic at birth. Audiological follow-up findings were available for 44 children, and sensorineural hearing loss was detected in 10 of them. For each congenitally infected child born, the type of maternal CMV infection was determined by evaluating the results of the serological screening (11). Among the 55 congenitally infected neonates, 25 were born after primary maternal CMV infection and 13 were born after recurrent maternal infection; for 17 of them, the type of maternal infection could not be determined.

Control infants. Each center individually determined the specificity of the method that it used with more than 50 Guthrie cards containing blood from infants in the absence of congenital infection. The specificity in both centers was 100% (15). For this study, 12 more Guthrie cards with blood samples from children proven to be cCMV negative were used as negative controls. The absence of congenital infection was confirmed by a negative urine culture for CMV in the first 7 days after birth.

Nucleic acid extraction by manual phenol-chloroform method (center 1). CMV DNA was extracted from a whole single spot (diameter, approximately 1 cm). The blood filter spot was transferred into a 2-ml tube containing 400 μ l of extraction buffer (Tris-HCl [50 mM, pH 7.5], NaCl [50 mM], EDTA [10 mM], sodium dodecyl sulfate [0.5%], proteinase K [10 mg/ml]). The DBSs were completely submerged in the extraction buffer and incubated at 56°C for 1 h. The supernatant was then purified by phenol-chloroform extraction and ethanol precipitation and was resuspended in 25 μ l of DNase-free buffer (Tris-HCl [10 mM], EDTA [1 mM]) and was stored at -20°C until use (15).

Nucleic acid extraction by automated easyMAG method (center 2). Nucleic acid extraction was performed by using the NucliSens easyMAG automated extraction platform (bioMérieux, Boxtel, The Netherlands), according to the

* Corresponding author. Mailing address: Department of Microbiology, UZ Brussel, Laarbeeklaan 101, Brussels 1090, Belgium. Phone: 32 2 4775002. Fax: 32 2 4775015. E-mail: anne.naessens@uzbrussel.be.

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TABLE 1. Rates of CMV detection on Guthrie cards with blood from congenitally infected and noninfected neonates according to the extraction and amplification protocols used

PCR protocol	No. of samples positive/no. tested (%)	
	Congenitally infected neonates	Not congenitally infected neonates
Manual extraction + conventional PCR ^a	36/55 (66)	0/12 (0)
easyMAG extraction + real-time PCR ^b	40/55 (73)	0/12 (0)
Manual extraction + real-time PCR	45/55 (82)	0/12 (0)
easyMAG extraction + conventional PCR	25/55 (45)	0/12 (0)

^a Methods routinely performed by center 1.^b Methods routinely performed by center 2.

manufacturer's recommendations. Briefly, a whole spot (diameter, approximately 1 cm) was transferred into 2 ml of NucliSens lysis buffer. The blood was eluted by gently rocking the sample for 30 min at 37°C. The supernatant was clarified by centrifugation at 250 × *g* for 1 min and was transferred to the sample vessel. Total nucleic acids were then extracted with the off-board protocol (Generic, version 1.06) on the NucliSens easyMAG platform, eluted in 25 µl of elution buffer, and stored at −20°C until use.

CMV DNA amplification by conventional PCR (center 1). The extracted DBS DNA (5 µl) was amplified by two distinct PCR assays (15). The first PCR (PCR1) used primers specific for the noncoding US8 region (primers 5'-GGA TCC GCA TGG CAT TCA CGT ATG T-3' and 5'-GAA TTC AGT GGA TAA CCT GCG GCG A-3'). The PCR conditions consisted of 1 cycle of 15 min at 95°C, followed by 34 cycles of 30 s at 95°C, 1 min at 55°C, and 50 s at 72°C and a final cycle of 10 min at 72°C. The second PCR (PCR2) is a nested PCR with primers specific for the glycoprotein H (UL75) region (outer primer set, 5'-AGG TAT TGA CAG ATC AAT GG-3' and 5'-CTC CTT CTC TCG GGT GTA AC-3'; inner primer set, 5'-CCA CCT GGA TCA CGC CGC TG-3' and 5'-TGG TGT TTT CAC GCA GGA A-3'). For both PCRs, the conditions were 1 cycle of 10 min at 95°C, followed by 34 cycles of 45 s at 95°C, 1 min at 55°C, and 50 s at 72°C and a final cycle of 10 min at 72°C. The amplified products of PCR1 (409 bp) were separated on a 2% agarose gel, and their specificity was confirmed by specific hybridization (with probe 5'-G GGG TAC ACA GGC GTG CCA CCG CAG ATA GTA AGC GC-3'). The amplified products of PCR2 (215 bp) were visualized on a 2% agarose gel.

The result was reported as positive when the result of at least one of the two PCRs was positive. The detection limit of this method was found to be 2×10^3 CMV DNA copies/ml of whole blood.

CMV DNA amplification by real-time PCR (center 2). For amplification of CMV DNA by real-time PCR, primers pp5549s and pp812as (6) and probes 5'-6-carboxyfluorescein-ACGTGGCACTGCGGCACGTGGT-6-carboxytetramethylrhodamine-3' were defined in the UL83 region. The PCR was performed in a 50-µl reaction mixture consisting of 25 µl Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 2 mM MgCl₂, 300 µM deoxynucleoside triphosphates, 0.2 µM (each) primer and probe, 1.25 U AmpliTaq Gold (Applied Biosystems), and 5 µl of DNA. The PCR conditions consisted of 1 cycle of 3 min at 95°C, followed by 50 cycles of 30 s at 95°C and 30 s at 60°C. Amplification, detection, and analysis were performed with the iCycler iQ real-time detection system (Bio-Rad).

If the CMV DNA amplification was negative, the PCR was repeated with the stored DNA extract.

The detection limit of this method was found to be 9.4×10^3 CMV DNA copies/ml of whole blood.

β-Globin DNA amplification by PCR. In both centers the quality of extraction as well as inhibition was monitored by amplification of the human β-globin gene. The conditions used to amplify the β-globin gene were identical to those used for the PCR amplification of CMV DNA, which allowed both reactions to be carried out simultaneously. Primers PCO4 and GH20 (13) and probes 5'-6-carboxyfluorescein-AACGGCAGACTTCTCCTCAGGAGT-6-carboxytetramethylrhodamine-3' were used.

Transport of extracted DNA. For evaluation of the extraction method, the DNA extracted from each blood spot was evaluated by the amplification method used by the collaborating center. For that purpose, the extracted DNA was transported on dry ice to the collaborating center.

Statistical methods. Statistical differences were calculated by the McNemar test (for differences between the different PCR protocols), Fisher's exact test (for

TABLE 2. Positivity rate according to type of maternal infection

PCR protocol	No. of samples positive/no. tested (%)	
	Primary maternal infection	Recurrent maternal infections
Manual extraction + conventional PCR ^a	17/25 (68)	7/13 (54)
easyMAG extraction + real-time PCR ^b	21/25 (84)	7/13 (54)
Manual extraction + real-time PCR	21/25 (84)	10/13 (77)
easyMAG extraction + conventional PCR	14/25 (56) ^c	2/13 (15) ^c

^a Methods routinely performed by center 1.^b Methods routinely performed by center 2.^c *P* = 0.036.

the sensitivity of the PCR in relation to the type of maternal infection), and logistic regression analysis (for sensitivity related to the period of storage of the Guthrie cards and gestational age at the time of the maternal primary infection).

RESULTS

Among the 55 Guthrie cards with blood from neonates with cCMV infections, 36 (66%) were positive at center 1 and 40 (73%) were positive at center 2 (*P* > 0.05). In nine instances, the PCR result was negative at both centers. For center 1, 24 samples were positive by the two PCRs and 12 other samples were positive by only one PCR. For center 2, the first PCR was positive for 32 of the samples, and repeat testing of the initially negative samples resulted in the detection of 8 more positive samples. None of the 12 Guthrie cards with blood from non-congenitally infected neonates were found to be positive.

The rates of detection of CMV in blood from congenitally infected and noninfected neonates on Guthrie cards according to the extraction and amplification protocols are summarized in Table 1. When DNA was extracted from the DBSs by the manual phenol-chloroform method, 45 yielded a positive result by the real-time PCR amplification protocol, whereas CMV DNA amplification by conventional PCR detected only 36 positive cases (*P* = 0.004). When CMV DNA was extracted from the DBSs by the automated easyMAG method, 40 were positive by real-time PCR amplification and only 25 were positive by the conventional PCR (*P* = 0.001).

The mean cycle threshold (*C_T*) values ± standard deviations of CMV DNA amplification obtained by the real-time PCR after the use of the manual extraction method and the automated easyMAG method were 36.6 ± 2.9 (range, 33.63 to 44.02) and 38.8 ± 1.4 (range, 28.84 to 42.87), respectively. The *C_T* value of the β-globin DNA amplification obtained by the real-time PCR method after the use of the manual extraction method (mean, 23.8 ± 1.9) was 3.1 lower than the *C_T* value obtained after the use of the automated easyMAG method (mean, 26.9 ± 1.4).

When the detection rate was analyzed according to the type of maternal infection (primary or recurrent), a trend toward a higher rate of detection of cCMV in neonates whose mothers had primary infections was seen. However, this difference was significant only for the easyMAG extraction method combined with conventional PCR (*P* = 0.036) (Table 2). No difference in the rate of detection of cCMV in infants born after maternal primary infection was seen when the estimated gestational age

when the maternal primary infection took place was taken into account.

Analysis of the results in relation to the length of storage of the cards did not reveal a significant difference in the detection rate after prolonged storage: the mean lengths of storage of the cards were 70 and 67 months for cards with positive results and 73 and 78 months for cards with negative results ($P > 0.05$) with the tests routinely performed at center 1 and center 2.

Among the 10 infants who developed adverse sequelae due to the congenital infection, CMV was detected in the DBSs of 7 (70%) at both laboratories. Among the 34 children without adverse sequelae, CMV was detected in the DBSs of 21 (62%) and 26 (76%) at center 1 and center 2, respectively. When these clinical data were taken into account, no significant difference in the detection rates was found ($P > 0.05$).

DISCUSSION

Blood stored as DBSs on Guthrie cards is taken at birth and may be stored for years until it is processed. Initially designed for the mass screening for phenylketonuria (7), these DBSs are now used for many different epidemiological purposes (12). In recent years they have been used for the retrospective diagnosis of cCMV infections in neonates (1, 2, 4, 9, 16).

In our study, detection rates of between 45 and 82% were found, depending on the protocol used. Johansson et al. (9) and Yamamoto et al. (16) had detection rates of 81 and 71%, respectively. The highest detection rate was found by Barbi et al. (1), whose protocol had a 100% sensitivity and a 99% specificity. Different factors may play a role in the positivity rate found by the different authors: an important factor is the method used in the laboratory. In this study, different protocols were tested with DBSs obtained from stored Guthrie cards. The differences in detection rates may also be attributed to the extraction methods as well as the amplification methods used. The manual extraction method gave better results than the easyMAG extraction method, whereas real-time DNA amplification performed better than the conventional method. The C_T values obtained by the real-time PCR were relatively high, regardless of the extraction method used. However, the C_T values were lower after phenol-chloroform extraction than after easyMAG extraction, indicating the better performance of the phenol-chloroform extraction method. By combining the most sensitive extraction method with the most sensitive DNA amplification method, a detection rate of 82% was found. This emphasizes the importance of the extraction method and indicates that more work on the optimization of the protocol for the extraction of CMV DNA is needed. Longer elution times are sometimes used with DBSs for the detection of other infectious diseases. Evaluations with longer incubation times were performed at center 1 without an increase in sensitivity (data not published). The C_T values obtained by real-time PCR in this study were, however, comparable to those obtained by Scanga et al. (14), who found a mean C_T value of 39 ± 4 (range, 33 to 45) and a 100% sensitivity with DBSs from seven neonates with cCMV infections.

Another possible explanation for the variability in sensitivity between the different methods could be the low viral load in congenitally infected neonates (5, 8), necessitating the use of the most sensitive methods. Indeed, testing of DBSs with viral

loads at the detection limits of the methods may result in discrepant results.

Since neonates with symptomatic disease may have higher viral loads than asymptomatic neonates (10), the selection of samples from more symptomatic neonates may be another reason for the differences in the detection rates. In our study population, we evaluated consecutive cases of neonates with congenital infections in whom CMV infection was diagnosed during an epidemiological survey (11), and two (4%) of the neonates were symptomatic. Yamamoto et al. (16), who also studied consecutive cases, found a 71% detection rate. A 100% detection rate was found in the study of Barbi et al. (1); however, 94 (57%) of the 164 congenitally infected neonates were symptomatic, indicating the selection of more severe cases. However, when we analyzed the rate of detection of CMV from DBSs on Guthrie cards according to the appearance of auditory sequelae, no association was found. DBSs on Guthrie cards from children who developed sequelae were as frequently positive as DBSs from those who did not.

It is important to emphasize that duplicate or even triplicate testing may be necessary to reach maximal sensitivity. Barbi et al. (1) analyzed each sample in triplicate and achieved 100% sensitivity. In our study, duplicate testing of the same extract was performed in both centers and was used as the standard methodology. Duplicate testing increased the sensitivity from 44% to 66% for center 1 and from 58% to 73% for center 2.

Better detection rates were seen for neonates with congenital infections originating from a maternal primary infection during pregnancy, regardless of the methods used. When we take into account only the methods routinely performed in the laboratories, the detection rate was only 54% when samples from neonates whose mothers had recurrent infections were analyzed and was 68 to 84% when samples from neonates whose mothers had primary infections were analyzed. These differences did not attain significance, although the number of samples studied might have been too low to attain significance. However, the systematically higher detection rates for neonates with primary infections should be further investigated on the basis of a larger series of data.

Guthrie cards can be stored for very long periods without a loss of sensitivity. Positive results were obtained from cards stored for up to 130 months. The detection rate was not adversely affected by prolonged storage.

We can conclude that the use of blood samples taken in the first week of life and stored as DBSs on Guthrie cards may be a good tool for the mass screening of neonates for cCMV infections. In unselected neonates, sensitivities of up to 82% can be reached. The sensitivity seems to be higher for congenitally infected cases due to primary maternal CMV infections. It is important to have a good extraction method and a sensitive PCR. Although the easyMAG extraction method is less powerful than the manual method, it seems to be more suitable for mass screening purposes because of its automation. Unfortunately, for optimal sensitivity, duplicate amplification is mandatory, which will increase the cost if mass screening is taken in consideration.

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